RESEARCH LETTER

Ichthyophthirius multifiliis as a potential vector of *Edwardsiella ictaluri* in channel catfish

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Abstract

There is limited information on whether parasites act as vectors to transmit bacteria in fish. In this trial, we used *Ichthyophthirius multifiliis* and fluorescent *Edwardsiella ictaluri* as a model to study the interaction between parasite, bacterium, and fish. The percentage (23–39%) of theronts fluorescing after exposure to *E. ictaluri* was significantly higher than control theronts (~ 6%) using flow cytometry. Theronts exposed to *E. ictaluri* at 4×10^7 CFU mL^{-1} showed a higher percentage (~ 60%) of fluorescent theronts compared to those (42%) exposed to 4×10^3 CFU mL^{-1} at 4 h. All tomonts (100%) carried the bacterium after exposure to *E. ictaluri*. *Edwardsiella ictaluri* survived and replicated during tomont division. Confocal microscopy demonstrated that *E. ictaluri* was associated with the tomont surface. Among theronts released from tomonts exposed to *E. ictaluri*, 31–66% were observed with attached *E. ictaluri*. Sixty percent of fish exposed to theronts treated with 5×10^7 *E. ictaluri* mL^{-1} were positive for *E. ictaluri* at 4 h as determined by qPCR or fluorescent microscopy. Fluorescent *E. ictaluri* were observed on trophonts in skin and gill wet mounts of dead fish. This study demonstrated that Ich could vector *E. ictaluri* to channel catfish.

Introduction

In aquaculture systems, fish rarely encounter a single pathogen. Most often, fish are concomitantly infected by multiple disease agents (Shoemaker *et al.*, 2008). Parasitism has been demonstrated to enhance bacterial invasion where parasitic injuries serve as portals of entry (Buchmann & Lindenstrøm, 2002; Busch *et al.*, 2003; Bandilla *et al.*, 2006). Ahne (1985) reported that parasites *Argulus foliaceus* and *Piscicola geometra* served as mechanical vectors for spring viremia of carp virus (SVCV). Vijayan *et al.* (2005) reported that polychaete worms acted as vectors of white spot syndrome virus in the transmission of white spot disease to the shrimp *Penaeus monodon*. Cusack & Cone (1985) detected bacterial colonies on the surface of *Gyrodactylus* by scanning electron microscopy. However, they did not determine whether the bacteria were pathogenic to fish, and thus, the exact role of the bacteria was not clear.

Protozoan *Ichthyophthirius multifiliis* (Ich) and bacterium *Edwardsiella ictaluri* are two pathogens of cultured catfish, which cause high fish mortality (Paperna, 1972; Hawke *et al.*, 1998). Enteric septicemia of catfish (ESC), caused by the bacterium *E. ictaluri*, is responsible for approximately 50% of economic losses to catfish farmers in the United States (Klesius, 1993; Shoemaker *et al.*, 2009). *Edwardsiella ictaluri* is a gram-negative enteric pathogen in catfish, and outbreaks of ESC are seasonal, occurring mainly in spring and fall with a temperature range of 22–28 °C (Tucker & Robinson, 1990). Ichthyophthiriasis is a major parasitic disease of freshwater fish worldwide, caused by a ciliated protozoan Ich. The parasite life cycle consists of an infective theront, a parasitic trophont, and a reproductive tomont (Hines & Spira, 1974; Matthews, 2005; Dickerson, 2006). Mature tomonts leave the fish host, attach to a substrate, and undergo multiple divisions to produce hundreds to thousands of infective theronts. Theronts swim actively in water in search of new fish hosts (Dickerson, 2006). The temperature ranges of ESC outbreaks overlap the optimum temperature window of Ich infection at 22–24 °C (Matthews, 2005; Dickerson, 2006). In 2002, 50.5% and 44.3% of all catfish operations (approximately 1000 total in the USA) had losses caused by ESC and by Ich (white spot), respectively (Hanson *et al.*, 2008).

The ability of parasites to enhance mortality because of bacterial diseases is presently receiving attention in aquaculture research. However, there is limited information on whether parasites act as vectors to transmit pathogenic bacteria in fish. To prevent and manage bacterial diseases in aquaculture, it is important to understand the potential of parasites to vector bacteria in fish. Parasites may easily transmit pathogenic bacteria from one fish to another within high-density fish populations on farms. In this trial, we used Ich–*E. ictaluri* as a model to study the interaction between the parasite, the bacteria, and the fish host. This study tested the hypothesis that Ich can vector *E. ictaluri* into channel catfish, *Ictalurus punctatus*. We further established that the bacteria were associated with the surface of the parasite. The bacteria multiplied and were transferred as the parasite divided.

Materials and methods

Fish and parasite

Channel catfish (industry pool strain) were obtained from disease-free stock from the USDA-ARS Catfish Genetic Research Unit, Stoneville, MS, and reared to the experimental size in indoor tanks at the USDA, Aquatic Animal Health Research Unit, Auburn, AL. *I. multifiliis* (ARS 10-1 strain) originally isolated from infected tropical pet fish was maintained by serial transmission on channel catfish held in 50-L glass aquaria, and theronts were cultured as described by Xu *et al.* (2000).

Bacterial strain culture and transformation

Edwardsiella ictaluri AL-93-58 was transformed with the pZsGreen vector (Clontech, Mountain View, CA) by Russo *et al.* (2009). The pZsGreen is a pUC19-derived prokaryotic expression vector that encodes a green fluorescent protein (ZsGreen) derived from the *Zoanthus* sp. and encodes ampicillin resistance. The transformed *E. ictaluri* were confirmed by PCR using *E. ictaluri*-specific primers (Russo *et al.*, 2009).

Edwardsiella ictaluri attachment to theronts

Twenty-four 15-mL tubes were filled with theront solution at 8 mL per tube. *Edwardsiella ictaluri* was added to theronts as follows: (1) 0 CFU mL^{-1} (no bacteria); (2) 4×10^3 CFU mL^{-1}; (3) 4×10^5 CFU mL^{-1}; and (4) 4×10^7 CFU mL^{-1}. Theronts in 12 tubes were exposed to *E. ictaluri* for 1 h and the remaining 12 tubes for 4 h. Triplicate tubes were used for each combination of *E. ictaluri* concentration and exposure time. At the end of each sampling time, formalin was added to each tube to fix theronts at 1% for 30 min. Theronts were washed with sterile water three times and harvested by centrifugation at 240 ***g*** for 3 min. The supernatant was discarded, and theronts were suspended in 0.5 mL sterile water in a flow cytometer tube. The number of theronts carrying fluorescent *E. ictaluri* was counted for each sample using the Coulter Epics flow cytometer (Beckman Coulter, Inc.) equipped with a 15 mW argon ion laser operating at 488 nm. Theronts without *E. ictaluri* exposure were included as negative controls. The percentage of theronts fluorescing was determined from ~ 1000 theronts in each sample.

Tomont exposure to *E. ictaluri* produced theronts carrying *E. ictaluri*

Fish infected with maturing tomonts were anesthetized with 150 mg L^{-1} tricaine methanesulfonate (MS-222) and rinsed in tank water, and the skin was gently scraped to dislodge the parasites. Four six-well plates were filled with 300 tomonts $well^{-1}$. Each plate had three treatments with two wells per treatment in all plates. *Edwardsiella ictaluri* was added to wells in each plate as follows: (1) 0 CFU mL^{-1}; (2) 4×10^5 CFU mL^{-1}; and (3) 4×10^7 CFU mL^{-1}. Tomonts were exposed to *E. ictaluri* at room temperature for 2 h. Then, the bacterial suspension and unattached tomonts were removed from each well. Fresh tank water was added to each well to wash (three times) the attached tomonts and remove suspended bacteria. After washing, 30 mL fresh tank water was added to each well and incubated at 22 ± 2 °C. One plate was sampled at 2, 4, 8, or 24 h postexposure to *E. ictaluri*. At the end of each sampling time, the attached tomonts (2–8 h) or theronts (24 h) were harvested and fixed with 1% formalin. After washing three times with clean water, one drop of tomont or theront sample and one drop of Gel/MountTM aqueous mounting medium (Sigma) were placed on a slide and covered with a cover slip. The slides were viewed with an Olympus BX41 fluorescence microscope and photographed with an Olympus DP70 digital microscope camera.

Confocal laser scanning microscopy

The distribution of *E. ictaluri* on the parasite (tomont specimens) was examined using a Zeiss Axioplan 2 microscope (Göttingen, Germany) fitted with a Bio-Rad Radiance 2000 confocal scan head. Laser scanning was controlled using Lasersharp 2000 software (Bio-Rad). Series of optical sections of 4 μm from top to bottom of tomonts were made as *z*-series and digital images collected.

Infection trial

Three 1-L beakers were filled with 200 mL of theront solution each at a concentration of 6800 theronts mL^{-1}. *Edwardsiella ictaluri* was added to each beaker as follows: (1) 0 CFU mL^{-1}; (2) 4×10^5 CFU mL^{-1}; and (3) 4×10^7 CFU mL^{-1}. After exposure to *E. ictaluri* for 1 h, theronts were harvested by centrifugation in 50-mL tubes at 240 ***g*** for 3 min and the supernatant discarded. Theronts were then washed (three times) with fresh tank water and centrifuged, and the supernatant was discarded to remove nonadherent bacteria. After washing, theronts were suspended in 100 mL tank water and enumerated with a Sedgwick-Rafter cell (Xu *et al.*, 2000).

Six 2-L beakers were used with 1 L water and 30 channel catfish fingerlings distributed in each container. The fish (3.3 ± 0.5 cm in length and 0.3 ± 0.1 g in weight) were acclimated to laboratory conditions 3 days prior to the trial. Water in each beaker was reduced to 0.5 L. The theronts exposed to various concentrations of *E. ictaluri* were added to each beaker at 1000 theronts $fish^{-1}$ (two beakers for each treatment). Five fish were sampled from each beaker at 4 h, 1 day, and 2 days post-theront exposure. The remaining 15 fish in each beaker were monitored for mortality. Each sampled fish was put in a 1.5-mL microcentrifuge tube, labeled, and washed with sterile water three times. Each fish was homogenized after adding 0.5 mL sterile water to a clean microcentrifuge tube using a 1.5-mL pellet pestle. Half of the fish tissue from each sample was transferred to a 15-mL tube with 5 mL brain heart infusion (BHI) broth containing 100 μg mL^{-1} ampicillin and incubated at 28 °C for 24 h with shaking. The pZsGreen-transformed *E. ictaluri* was able to grow in BHI with ampicillin, but other autochthonous bacteria were inhibited. The presence of *E. ictaluri* was examined by florescence microscopy at 24 h postculture. The remaining fish tissue was frozen at −20 °C for DNA extraction and used for qPCR.

Genomic DNA isolation from fish tissues

The tissues preserved at −20 °C were used to extract DNA and quantitate *E. ictaluri* with qPCR. Total genomic DNA of *E. ictaluri* in fish tissues was extracted by the DNeasy Tissue kit and eluted into 200 μL water according to the manufacturer's instructions. DNA yield and purity were determined using a Nanodrop ND-1000. The gDNA was stored at −20 °C until use.

Quantitative real-time PCR

One-step qPCR was performed as described by Bilodeau *et al.* (2003) using *E. ictaluri*-specific primers (forward 5′-ACTTATCGCCCTCGCAACTC-3′ and reverse 5′-CCTCTGATAAGTGGTTCTCG-3′) and a dual-labeled probe (5′-CCTCACATATTGCTTCAGCGTCGAC-3′). Reactions were completed using an Applied Biosystems 7500 with the following conditions: 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Extracted DNA from fish tissue (1 μL) was used as template in qPCR, and the DNA concentration in fish tissue was determined via the standard curve [threshold cycle (C_t) values vs. DNA concentration of *E. ictaluri*]. For standards, 10-fold serial dilutions from 5 ng μL^{-1} to 5 fg μL^{-1} of the gDNA of *E. ictaluri* were made in sterile water. As 1 μL of eluted sample was run in qPCR, the amount of bacterial DNA in each milligram of tissue was equal to: bacterial DNA concentration (pg μL^{-1}) × eluted volume/tissue weight (mg). Bacterial DNA in each milligram of tissue was calculated as genome equivalents per milligram of tissue (GEs mg^{-1}) based on the genome size of *E. ictaluri* = 3.8 fg $cell^{-1}$ (Bilodeau *et al.*, 2003).

Statistical analysis

Data were analyzed with SAS software (SAS, 1989). Percentages of theronts vectoring *E. ictaluri* were analyzed with Duncan's multiple range test of the general linear model (GLM) procedure. The correlation between the bacterial concentrations and numbers of theront carrying *E. ictaluri* or between bacterial concentrations used to treat theronts and numbers of fish positive for *E. ictaluri* was evaluated with Spearman correlation. Probabilities of 0.05 or less were considered statistically significant.

Results

E. ictaluri attached to theronts

Using flow cytometry, control theronts not exposed to *E. ictaluri* showed 6–8% fluorescing theronts, indicating low background autofluorescence (Table 1). Theronts exposed to *E. ictaluri* demonstrated significantly higher counts ($P < 0.05$) compared to control theronts. Almost 60% of theronts exposed to *E. ictaluri* at 4×10^7 CFU mL^{-1} were fluorescent as compared to 42% exposed to 4×10^3 CFU mL^{-1} 4 h postexposure to fluorescent *E. ictaluri*. There was a strong correlation between the *E. ictaluri* concentration and the number of fluorescing theronts (correlation coefficient = 0.75, $P < 0.01$). Theronts exposed to *E. ictaluri* for a longer duration (4 h) at all three concentrations also demonstrated a higher percentage of fluorescent theronts as compared to those exposed for 1 h.

Table 1. Flow cytometry results indicating the numbers of theronts positive for *Edwardsiella ictaluri* 1 or 4 h postexposure to the bacterium

Time post exposure	Concentration of *E. ictaluri* (CFU mL^{-1})	Theronts counted	Fluorescent theronts	% of theronts positive for *E. ictaluri*
1 h	0	931 ± 43	53 ± 5	5.7 ± 0.3^{a}
	4×10^3	992 ± 3	233 ± 18	23.5 ± 1.8^{b}
	4×10^5	1000 ± 30	320 ± 11	32.0 ± 12.2^{c}
	4×10^7	956 ± 40	321 ± 12	39.0 ± 5.3^{c}
4 h	0	839 ± 95	67 ± 19	7.7 ± 1.5^{a}
	4×10^3	999 ± 6	421 ± 37	42.1 ± 3.7^{d}
	4×10^5	1000 ± 3	513 ± 11	51.3 ± 1.1^{e}
	4×10^7	986 ± 6	597 ± 40	59.7 ± 4.0^{f}

Each value is the mean of three samples (± SEM). Within a given column, means followed by different superscript letters are statistically different ($P < 0.05$).

Tomonts exposed to *E. ictaluri* produced theronts carrying *E. ictaluri*

No fluorescent bacteria were observed on control tomonts (i.e. not exposed to *E. ictaluri*). All tomonts (100%) demonstrated fluorescent bacteria 2–8 h postexposure to *E. ictaluri* at 5×10^5 or 5×10^7 CFU mL^{-1} (Table 2). Tomonts exposed to *E. ictaluri* at 5×10^7 CFU mL^{-1} showed more bacteria than those exposed to *E. ictaluri* at 5×10^5 CFU mL^{-1} (Fig. 1). The bacterial number also increased from 2 to 8 h postexposure (Fig. 1), suggesting bacterial replication. After 24 h, most tomonts divided into several hundred tomites and released infective theronts. Among those theronts, 31.2% and 66.4% were observed to have fluorescent bacteria attached following tomont exposure to *E. ictaluri* at 5×10^5 CFU mL^{-1} or 5×10^7 CFU mL^{-1}, respectively (Table 2). Theronts produced from tomonts exposed to *E. ictaluri* at 5×10^7 CFU mL^{-1} showed more fluorescent bacteria than those exposed to *E. ictaluri* at 5×10^5 CFU mL^{-1} (Fig. 1). *Edwardsiella ictaluri* survived and grew during the tomont division. Fluorescent bacteria were seen on tomonts and theronts collected at all sampling times (Fig. 1).

Distribution of *E. ictaluri* on Ich tomonts

The location of *E. ictaluri* was examined from *z*-series optical sections of tomonts 2 h postexposure to *E. ictaluri* at 5×10^7 CFU mL^{-1} by confocal microscopy (Fig. 2). Many fluorescent bacteria were seen on the surface of the tomont (1a–1b). The numbers of fluorescent bacteria gradually decreased on deeper sections of tomonts (1c–1d). No bacteria were observed in the middle section of tomonts (2a–2b) except on the margins. Then, the numbers of fluorescent bacteria gradually increased on the bottom surface of tomont (2c–2d) and reached high numbers of fluorescent bacteria at the bottom section of tomont (3a-3b). The numbers of the bacteria decreased as the section passed completely through the tomont (3c–3d).

Infection trial

Fish showed mortality 1 day postexposure to theronts. Mortalities were 13.3%, 13.3%, and 23.4% for fish exposed to theronts only, theronts treated with 5×10^5 *E. ictaluri* mL^{-1}, and theronts treated with 5×10^7 *E. ictaluri* mL^{-1}, respectively. At 2 days postexposure, fish cumulative mortalities were 36.7%, 40.0%, and 60.0% for fish exposed to theronts only, theronts treated with 5×10^5 *E. ictaluri* mL^{-1}, and theronts treated with 5×10^7 *E. ictaluri* mL^{-1}, respectively. Trophonts were detected in skin and gill of wet mounts from dead fish (Fig. 3a). Fluorescence microscopy demonstrated *E. ictaluri* on or near trophonts (Fig. 3b).

Fifty percent, 70% and 40% of fish were positive for *E. ictaluri* by qPCR at 4 h, 1 day, and 2 days, respectively, postexposure to theronts treated with 5×10^5 *E. ictaluri* mL^{-1} (Table 3). When fish were exposed to theronts treated with 5×10^7 *E. ictaluri* mL^{-1}, 100%, 90%, and 60% of fish were *E. ictaluri* positive at 4 h, 1 day, and 2 days, respectively. A correlation was noted between theront *E. ictaluri* exposure concentration and the numbers of fish positive for *E. ictaluri* (correlation coefficient = 0.68, $P < 0.01$). Fish exposed to theronts

Table 2. Number of tomonts (2–8 h) or theronts (24 h) positive for *Edwardsiella ictaluri* (P) observed under fluorescent microscope. The tomonts were exposed to *E. ictaluri* for 2 h and then underwent division for different periods of time

Concentration of *E. ictaluri* (CFU mL^{-1})	Hour 2		Hour 4		Hour 8		Hour 24	
	P/total	% P	P/total	% P	P/total	% P	P/total	% P
0	0/25	0	0/31	0	0/30	0	0/261	0
5×10^5	41/41	100	45/45	100	30/30	100	77/247	31.2
5×10^7	36/36	100	40/40	100	45/45	100	184/277	66.4

P, number of tomonts or theronts positive for *E. ictaluri;* total, tomonts or theronts examined; % P, percentage of tomonts or theronts positive for *E. ictaluri*.

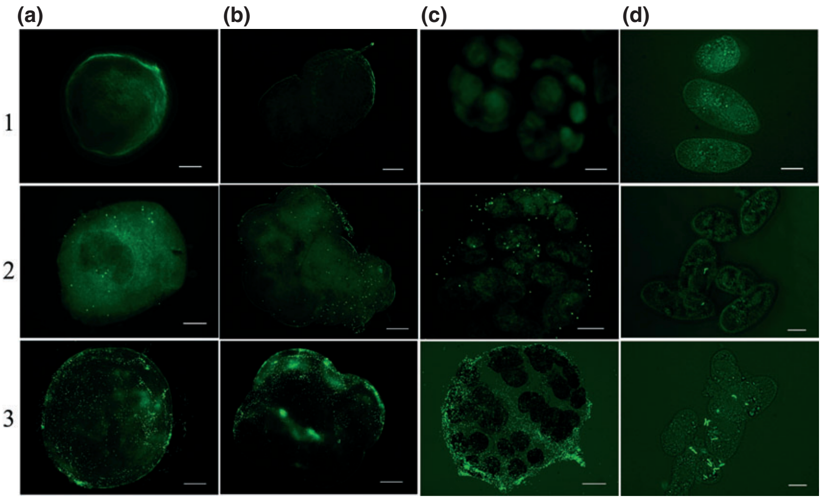

Fig. 1. Fluorescence micrographs of tomonts during reproductive stage. Row 1: tomonts exposed to no bacteria; row 2: tomonts exposed to *Edwardsiella ictaluri* at 4×10^5 CFU mL^{-1}; row 3: tomonts exposed to *E. ictaluri* at 4×10^7 CFU mL^{-1}. Column a, b, c, and d are tomonts at 2, 4, 8, or 24 h postexposure to *E. ictaluri.* Bar in column a, b, c (tomonts) = 100 μm and bar in column d (theronts) = 20 μm.

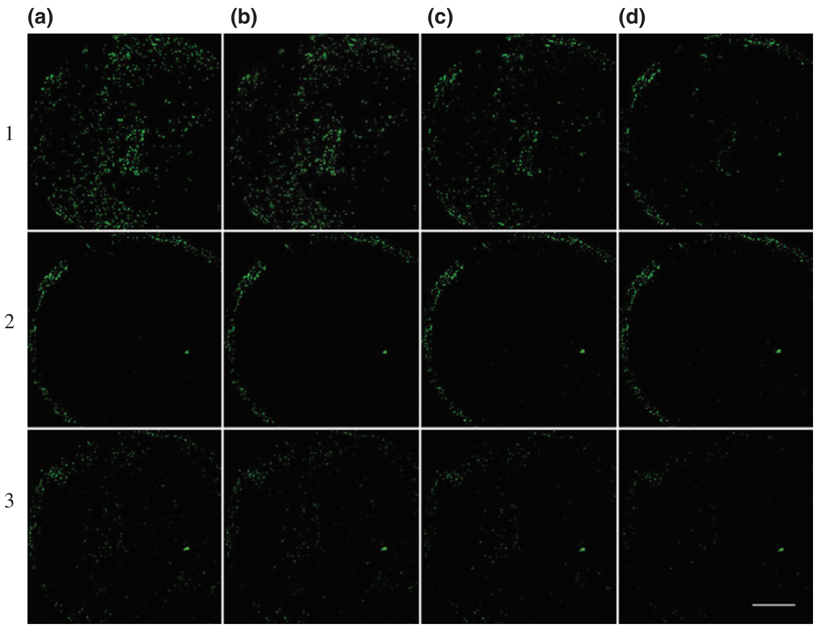

Fig. 2. Confocal laser scanning images of a tomont 2 h postexposure to *Edwardsiella ictaluri* at 4×10^7 CFU mL^{-1}. The tomont was optically scanned at 4 μm per section from top and arranged in every other section. Bar = 135 μm.

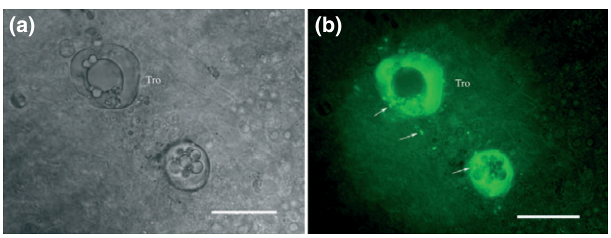

Fig. 3. Light and fluorescence micrographs of trophonts in a wet mount of gills from a dead channel catfish 4 h postexposure to theronts treated with 5×10^7 *Edwardsiella ictaluri* mL^{-1}. (a) Two trophonts (Tro) observed on gills of catfish under normal light (bar = 65 μm); (b) the same view observed using fluorescence light, green *E. ictaluri* (arrow) were seen on or nearby two trophonts (bar = 65 μm). Trophonts showed weak autofluorescence.

treated with 5×10^7 *E. ictaluri* mL^{-1} showed significantly higher GE in tissues ($P < 0.05$) than fish exposed to theronts treated with 5×10^5 *E. ictaluri* mL^{-1} (Table 3). The fish showed a 170.8, 95.3, and 77.2 GE mg^{-1} of tissues at 4 h, 1 day, and 2 days, respectively, postexposure to theronts treated with 5×10^7 CFU *E. ictaluri* mL^{-1}. No *E. ictaluri* was detected by qPCR in fish exposed to theronts only (Table 3).

Fish tissues were incubated in BHI for 24 h and then examined for *E. ictaluri* using florescence microscopy. Sixty percent, 90%, and 70% of fish exposed to theronts treated with 5×10^5 *E. ictaluri* mL^{-1} showed fluorescent bacteria at 4 h, 1 day, and 2 days, respectively. Fish were 100%, 100%, and 90% positive for *E. ictaluri* at 4 h,

Table 3. Number of fish samples positive for *Edwardsiella ictaluri* at different times after exposure to theronts treated with the bacterium. Each fish was homogenized, and the genomic DNA was extracted from half of the sample. Two samples from each fish were used to determine the genome equivalent of *E. ictaluri* with qPCR

Concentration of *E. ictaluri* (CFU mL^{-1})	Hour 4			Day 1			Day 2		
	P/N	% P	GE ± SEM	P/N	% P	GE ± SEM	P/N	% P	GE ± SEM
0	0/20	0^a	0 ± 0^a	0/20	0^a	0 ± 0^a	0/20	0^a	0 ± 0^a
5×10^5	10/20	50^b	48.7 ± 17.4^a	14/20	70^b	55.4 ± 13.7^b	8/20	40^b	29.0 ± 9.6^a
5×10^7	20/20	100^c	170.8 ± 27.2^b	18/20	90^b	95.3 ± 11.7^c	12/20	60^b	77.2 ± 18.1^b

P, number of fish positive for *E. ictaluri; N*, number of fish examined; % P, percentage of fish positive for *E. ictaluri*; GE ± SEM, genome equivalent ± standard error of mean.
Within a given column, means followed by different superscript letters are statistically different ($P < 0.05$).

1 day, and 2 days, respectively, postexposure to theronts treated with 5×10^7 *E. ictaluri* mL^{-1}. There was a correlation between the *E. ictaluri* concentration that theronts were exposed to and the numbers of fish positive for *E. ictaluri* (correlation coefficient = 0.79, $P < 0.01$). No fluorescent bacteria were detected in fish exposed to theronts only (Table 4). There was a significant correlation between the numbers of fish positive for *E. ictaluri* by qPCR and those positive by fluorescent microscopy (correlation coefficient = 0.80, $P < 0.01$).

Discussion

Previous studies by our group and others have demonstrated that parasitism enhances mortality in fish coinfected with bacteria regardless of the order of infection (i.e. parasitism followed by bacterial exposure or vice versa). Our hypothesis in this study was that Ich, a ciliated protozoan parasite, could vector *E. ictaluri*, a bacterial pathogen, into channel catfish. Our results using fluorescent *E. ictaluri* demonstrated that the bacteria attached to the Ich reproductive and infective stages (tomonts and theronts). Confocal microscopy further demonstrated a close association of *E. ictaluri* with the surface of Ich and that the bacteria were not internalized. In a previous study, we demonstrated using lectins that surface carbohydrates are present on Ich theronts (Xu *et al.*, 2001). Soybean agglutinin and lentil agglutinin were the most effective at binding Ich theronts, suggesting that the sugar molecules present were D-galactose, D-mannose, D-glucose, and *N*-acetylgalactosamine. The presence of receptors for D-galactose (Wolfe *et al.*, 1998) and D-mannose (Ainsworth, 1993) on the surface of *E. ictaluri* has been demonstrated. We hypothesize that the interaction between the *E. ictaluri* lectin-like receptors and Ich surface D-galactose or D-mannose resulted in binding. Further studies are needed to confirm this hypothesis. Nevertheless, the binding of *E. ictaluri* did not inhibit the replication of Ich tomonts and/or the movement and attachment of Ich theronts to the host.

Edwardsiella ictaluri survived and appeared to replicate on different stage(s) of tomonts. After substrate attachment, tomonts divide from a single cell to hundreds of daughter tomites and differentiate into infective theronts. The tomonts at 8 h postexposure to *E. ictaluri* showed more fluorescent bacteria compared to those at 2 h, suggesting bacterial replication. *Edwardsiella ictaluri* was mainly located on the surface of tomonts when observed under fluorescent microscope. The results were confirmed using a confocal microscope by scanning different layers of tomonts from top to bottom. The initial exposure concentrations of *E. ictaluri* influenced the numbers of fluorescent bacteria adhering to tomonts with the high concentration of *E. ictaluri* showing more bacteria. After release from tomont cysts, more theronts (66.4%) were noted to carry *E. ictaluri* when tomonts were exposed to *E. ictaluri* at 5×10^7 CFU mL^{-1} than those exposed to 5×10^5 CFU mL^{-1}. The data suggest that the bacteria are passed directly to theronts during tomont division. Further studies are needed to demonstrate the exact mechanism of transfer.

Theronts with adherent *E. ictaluri* swam in water, contacted fish, and then penetrated into fish skin or gills. The fluorescent bacteria were detected in fish after exposure to theronts carrying *E. ictaluri* by qPCR and fluorescent microscopy. Both methods showed similar results

Table 4. Number of fish positive for *Edwardsiella ictaluri* at different times after exposure to theronts treated with *E. ictaluri*. Each fish was homogenized and incubated with BHI broth containing 100 μg mL^{-1} ampicillin overnight. The fluorescent *E. ictaluri* in fish sample was observed under fluorescent microscopy, and observation of any green fluorescent bacteria was scored positive

Concentration of *E. ictaluri* (CFU mL^{-1})	Hour 4		Day 1		Day 2	
	P/N	% P	P/N	% P	P/N	% P
0	0/10	0	0/10	0	0/10	0
5×10^5	6/10	60	9/10	90	7/10	70
5×10^7	10/10	100	10/10	100	9/10	90

P, number of fish positive for *E. ictaluri; N*, number of fish examined; % P, percentage of fish positive for *E. ictaluri*.

with a high correlation. qPCR demonstrated that fish exposed to theronts treated with *E. ictaluri* at high concentration showed higher *E. ictaluri* load (77–170 GE mg^{-1}) than fish exposed to theronts treated with low concentration of bacteria (29–55 GE mg^{-1}) from 4 h to 2 days. When examining dead fish for parasite infection, trophonts were observed on skin and gill wet mounts. Previously, Xu *et al.* (2000) found that trophonts rounded to an oval shape, began rotation, and created intercellular spaces via trophont motion. In this study, fluorescent *E. ictaluri* were clearly seen on or near trophonts (Fig. 3) that developed from the *E. ictaluri*-exposed theronts. The results suggest that *E. ictaluri* could then contact immune cells and be disseminated throughout the fish host. Early in the invasion process, some trophonts relocate to other infection sites of skin and gills in or on the same or different fish hosts (Xu *et al.*, 2000) and thus could potentially vector the bacteria to other fish.

In summary, this study provided evidence for the first time that Ich can vector *Edwardsiella ictaluri* into channel catfish. Ich theronts and tomonts carried *E. ictaluri* after exposure to the bacterium. Tomonts exposed to *E. ictaluri* could pass *E. ictaluri* to infective theronts released from the tomonts, and the theronts transmitted the bacterium to channel catfish. The vectoring ability of parasites is particularly important at fish farms because the introduction of parasites either from wild fish or from other farms could concomitantly involve the introduction and/or transmission of microbial diseases.

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